

Rotational Isomerism of the Phenylalanine Anion in Mixed Aqueous Solvents by Nuclear Magnetic Resonance¹

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The chemical shifts and coupling constants for the aliphatic protons of the phenylalanine anion in mixed aqueous solvents have been obtained as a function of temperature and solvent composition. In general, the vicinal coupling constants diverge as the mole fraction of the nonaqueous component increases. The effect increases in the series urea < ethylene glycol, acetamide < methanol < dimethyl sulfoxide < acetone, urea causing even a mild convergence with increasing concentration. For those solvent mixtures for which the vicinal coupling constants diverge, the divergence of the coupling constants with temperature is reduced. With aqueous urea as solvent, on the other hand, the results are almost identical with pure water. These variations are interpreted in terms of the conformational stability of the staggered rotamers, the less favorable rotamers becoming less stable as the proportion of the nonaqueous component increases. The rotamer stabilities appear to be generally related to solubility and dielectric properties of the mixed aqueous solvents, but no correlation can be found between variations in the coupling constants and the "water-like" character of these solvents.

In a series of investigations,⁴ the nmr spectra of the aromatic amino acids have been obtained in aqueous solution as a function of temperature and amino acid concentration. The results have been interpreted in terms of variations of the relative energies of the classical staggered rotamers. It appears that the rotamer energies are influenced by temperature and concentration-dependent solute-solute and solute-solvent interactions. The nature of these interactions is as yet unclear but, undoubtedly, the structure of water plays an important role. In order to vary systematically the properties of the water system, we have employed as solvents, mixtures of a variety of organic reagents with water at different concentrations. The effects of these mixed aqueous solvents on the rotational isomerism of the phenylalanine anion form the basis of the present communication.

Experimental Section

The nmr spectra were recorded on a Varian Associates DA-60 IL spectrometer,⁵ equipped with a variable temperature probe and operated at 60 MHz in the internal-lock, frequency-sweep mode. The frequency sweep was calibrated in the vicinity of each resonance peak by counting the difference between the fixed and swept oscillators. Line positions were calculated as the averages of at least four recordings taken with alternate upfield and downfield sweeps. Precision of the measurements was better than 0.1 Hz. The temperature in the probe was measured directly using a thermistor thermometer placed inside an empty nmr sample tube at the location of the transmitter-receiver coils. The probe assembly was fitted with a standard pressure cap through which the leads from the thermistor were led to a resistance bridge for measurement. The temperature could be measured to within 0.5° and remained stable to $\pm 1^\circ$ during the course of the nmr measurements.

The amino acids and the solvents were obtained commercially and were of the highest purity obtainable. Acet-

amide and urea were recrystallized from methanol solution; ethylene glycol was twice redistilled; the other compounds were used without further purification. The samples were prepared with the mole fraction of phenylalanine kept constant at 1/55.5 (corresponding to 1 mol of phenylalanine per liter of solvent for pure water) while the composition of the solvent was varied. Solvent compositions are reported in terms of mole fraction of the organic reagent with regard to the solvent. Sufficient NaOH was included in the mixture to ensure that the phenylalanine was in the anionic state. *tert*-Butyl alcohol (2% v/v) was added as an internal standard.

The solubilities were determined gravimetrically by the dry weight method described previously⁶ with the following modifications and exceptions. Additional water was added to the solutions of alanine and phenylalanine in dimethyl sulfoxide-water and the samples were dried by lyophilization before oven drying at 110°. For alanine in acetamide-water systems, water was first removed by rotary evaporation at 50°, the acetamide was separated by extraction with chloroform, and the alanine residue was finally dried at 110°. Runs carried out with known amounts of alanine showed that the method was quantitative. The concentration of phenylalanine in acetamide-water solutions was determined spectrophotometrically.⁶ As a further check on the dry weight method, the solubilities of alanine and phenylalanine in pure water were determined; results were in excellent agreement with those reported previously.⁶

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- (2) Research performed under tenure of an NRC-ARS Postdoctoral Research Associateship. Present address, McKesson Laboratories, Fairfield, Conn. 06430.
- (3) Agricultural Research Service, U. S. Department of Agriculture.
- (4) (a) J. R. Cavanaugh, *J. Amer. Chem. Soc.*, **89**, 1558 (1967); (b) *ibid.*, **90**, 4533 (1968); (c) *ibid.*, **92**, 1488 (1970).
- (5) Mention of commercial products does not constitute an endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.
- (6) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074 (1963).

Results and Discussion

Spectral analysis and the calculation of chemical shifts and coupling constants were performed as discussed previously.⁴ The results are presented in Table I.

Framework for the Interpretation of the Variations in the Vicinal Coupling Constants. It has become well established that the rotational isomerism of amino acids is adequately represented in the classical manner, that is, by an equilibrium mixture of the three staggered rotamers obtained by internal rotation about the α carbon- β carbon bond. The staggered rotamers are illustrated in Figure 1 for a substituted alanine. Since the internal rotation is sufficiently rapid, the observed coupling constants are weighted averages over those corresponding to the individual rotamers. The vicinal coupling constants are assumed to obey the form of the Karplus equation;⁷ that is, the coupling constants of protons gauche or trans to one another are assumed to have the same value regardless of the particular conformation. The averaged vicinal coupling constants, J_{12} and J_{13} , are then simply given by

$$\begin{aligned} J_{12} &= (a + c)J_g + bJ_t \\ J_{13} &= (a + b)J_g + cJ_t \end{aligned} \quad (1)$$

where a , b , and c are the normalized populations of rotamers A, B, and C, respectively, and J_g and J_t are the coupling constants of protons gauche or trans to one another. The populations are governed by the usual Boltzmann distribution; that is

$$a : b : c = e^{-F_A/RT} : e^{-F_B/RT} : e^{-F_C/RT} \quad (2)$$

where F_i is the appropriate rotamer free energy.

The rotational isomerism of the aromatic amino acids could not be explained simply in this fashion. The vicinal coupling constants diverged with increasing temperature in direct opposition to the expected trend toward equalization of the rotamer populations through the Boltzmann factor. The cause of the anomalous variations was interpreted⁸ as arising from changes in the rotamer energies themselves with temperature; the two less favorable rotamers, A and B, became less stable as the temperature increased. Concentration effects were also in evidence. Apparently, the rotamer energies were being influenced by temperature and concentration-dependent solute-solute and solute-solvent interactions.

Vicinal Coupling Constants as a Function of Composition and Temperature. The variations in the vicinal coupling constants for the phenylalanine anion as a function of the composition of the mixed aqueous solvents are illustrated in Figure 2.⁹ The values shown represent the data at 28° for all solvent systems except ethylene glycol. The viscosity of ethylene glycol at room temperature causes sufficient deterioration of spectral resolution that higher temperatures were required for accurate measurements. The data for pure water were interpolated from the data in ref 4a at 28°. The values for acetamide and ethylene glycol actually differ slightly even though they are shown as superimposable in Figure 2 for clarity of presentation.

In general, the coupling constants diverge with increasing fraction of the nonaqueous component. The effect is largest for acetone and ranges down through the series dimethyl sulfoxide, methanol, acetamide, ethylene glycol, and urea. The results for urea exhibit even a slight convergence with increasing urea concentration.

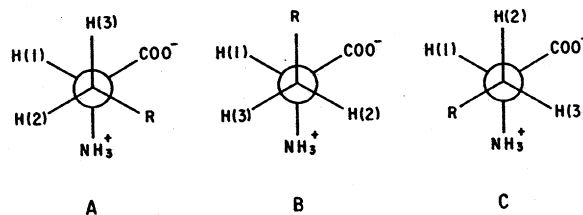


Figure 1. Newmann projections of the staggered rotamers of an R-substituted alanine (dipolar ion).

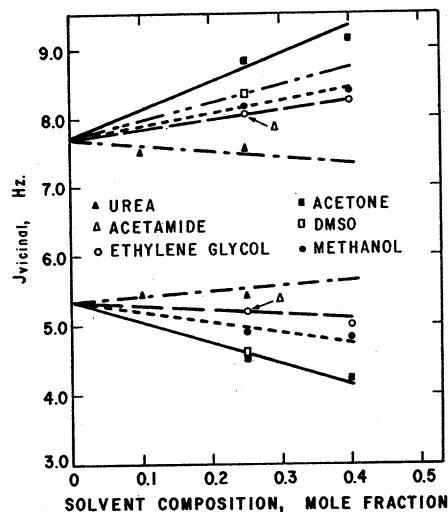


Figure 2. Variations of the phenylalanine anion vicinal coupling constants with solvent composition.

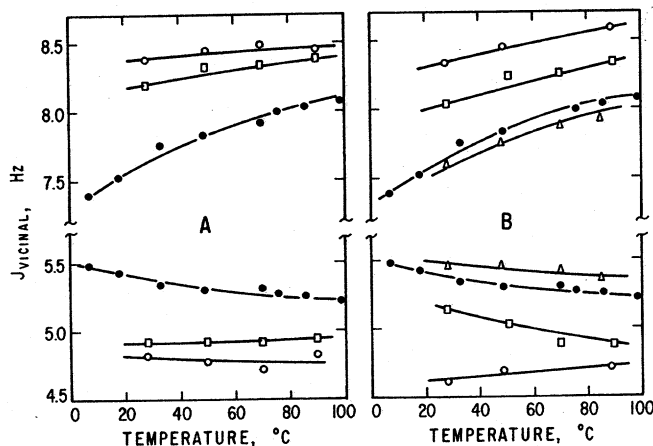


Figure 3. Temperature variations of the phenylalanine anion vicinal coupling constants in various solvent mixtures: ●, water; (A) □, 0.25 mol fraction methanol; ○, 0.4 mol fraction methanol; (B) △, 0.25 mol fraction urea; □, 0.25 mol fraction acetamide; ○, 0.25 mol fraction ethylene glycol.

The temperature variations of the coupling constants appropriate to some of these systems are shown in Figure 3. Included for comparison are the corresponding data for the phenylalanine anion in aqueous solution. The results for the alcohol-water system for two concentrations are displayed in Figure 3A, the outer curves representing the more concentrated alcohol system. Both curves exhibit

(7) M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959).

(8) Cf. ref 4 for details of the experimental results and for the argumentation leading to the interpretations outlined here.

(9) The lines shown are hand drawn and intended simply to distinguish trends.

TABLE I: Chemical Shifts^a and Coupling Constants^b for the Phenylalanine Anion^c

Nonaqueous component	Concn ^d	Temp ^e	J_g	J_v^f	$\Delta\nu_\alpha$	$\Delta\nu_\beta$		
Methanol- <i>d</i> ₄	0.25	28	-13.50	4.92	8.20	134.80	110.72	92.07
		50	-13.54	4.92	8.32	135.15	111.87	91.49
		70	-13.61	4.92	8.34	135.31	112.49	91.26
		90	-13.71	4.94	8.39	135.40	113.00	90.78
	0.4	28	-13.52	4.82	8.38	134.72	112.11	91.54
		50	-13.57	4.77	8.44	135.12	113.02	91.15
		70	-13.49	4.72	8.49	135.32	113.71	90.84
		90	-13.64	4.83	8.45	135.37	114.15	90.76
		Urea	0.1	28	-13.43	5.46	7.53	134.67
0.25	28		-13.47	5.46	7.60	136.19	106.67	94.22
	48		-13.50	5.45	7.75	136.30	107.94	93.41
	70		-13.60	5.42	7.87	136.81	109.23	93.41
	85		-13.70	5.36	7.92	136.83	109.60	92.61
Acetamide	0.25	28	-13.39	5.14	8.04	136.63	112.37	94.35
		51	-13.47	5.03	8.24	136.60	112.47	92.93
		70	-13.58	4.89	8.26	136.19	112.68	92.17
		90	-13.63	4.88	8.34	136.14	113.21	91.65
Dimethyl- <i>d</i> ₆ sulfoxide	0.25	28	-13.67	4.61	8.34	129.61	110.86	89.11
		49	-13.52	4.69	8.45	129.66	111.37	88.64
		89	-13.62	4.71	8.59	129.73	112.12	88.00
Acetone	0.25	28	-13.45	4.55	8.86	134.85	113.54	89.08
	0.40	28	-13.46	4.25	9.18	134.69	115.30	87.39
Ethylene glycol	0.25	52	-13.58	5.25	8.07	135.66	110.38	92.86
	0.40	52	-13.58	4.99	8.26	138.30	110.99	92.47

^a In Hz at 60-MHz downfield from *tert*-butyl alcohol (methyl resonance). ^b In Hz. ^c The concentration of the phenylalanine anion was kept constant at 1 mol per 55.5 mol of solvent mixture. ^d The concentration of the nonaqueous component in mole fraction units. ^e In °C. ^f The smaller of the two vicinal coupling constants is associated with the β proton resonance at lower field.

less divergence with increasing temperature than that shown for the water solution.

Some of the other systems are illustrated in Figure 3B. The curves for acetamide and dimethyl sulfoxide are similar to those for the methyl alcohol system with some variations. The smaller coupling constant for the acetamide-water system, for example, decreases slightly more rapidly with increasing temperature than that for pure water. On the other hand, the smaller coupling constant for the dimethyl sulfoxide system actually increases with increasing temperature. The urea system, for which there is little variation in the coupling constants with urea concentration, yields results remarkably close to those for pure water.

Qualitatively, the overall results may be summarized as follows: the larger the concentration divergent effect of the nonaqueous component on the averaged coupling constants, the more the anomalous temperature dependence appropriate to the aqueous solutions is attenuated. If the nonaqueous component has little effect on the coupling constants, the temperature dependence is not appreciably altered.

The variations in the coupling constants with solvent composition exhibited here must find their origin in variations in the rotamer populations. Even though it is conceivable that the proton-proton vicinal coupling constants in the staggered rotamers are themselves affected by changes in the solvent, the evidence against such a dependence on solvent medium is by now very secure, the best evidence coming from the invariance of the coupling con-

stants in conformationally rigid molecules.¹⁰ Furthermore, classical electrostatic theory has been very successfully applied to rotational isomerism¹¹ by treating the variations in proton-proton vicinal coupling constants as arising solely from medium effects on the rotamer energies.

Changes in the rotamer populations and in the relative rotamer energies can be related to changes in the coupling constants by means of eq 1 and 2. Numerical values are assigned to J_g and J_t , and the populations and energies calculated from the experimentally observed coupling constants.¹² According to this interpretation, C is the most favorable rotamer for the phenylalanine anion with a population at room temperature in pure water of about 0.5. As the coupling constants diverge with solvent composition, the less favorable rotamers, A and B, became even less favorable and rotamer C increases in population.

The temperature dependence for the mixed solvent systems is consistent with this interpretation. Those nonaqueous solvents that cause a divergence of the coupling constants bring about an increase in the relative energies of the unfavorable A and B rotamers. In these mixed solvents at room temperature, the rotamer energies are already increased, any further increase with temperature is correspondingly reduced, and the divergence of the cou-

(10) A. M. Ihrig and S. L. Smith, *J. Amer. Chem. Soc.*, **94**, 34 (1972); S. L. Smith and R. H. Cox, *J. Phys. Chem.*, **72**, 198 (1968); S. L. Smith and A. M. Ihrig, *J. Chem. Phys.*, **46**, 1181 (1967).

(11) R. J. Abraham and R. H. Kemp, *J. Chem. Soc. B*, 1240 (1971). This paper is the eleventh in the series. The series can be generated by referring in turn to each preceding issue.

pling constants with increasing temperature is thereby diminished.

The actual values calculated for the populations and the relative rotamer energies are of course contingent on the values chosen for J_g and J_t and on the assumption that a single J_g is adequate to describe the system. Nevertheless, interpretations developed here will be based on changes in these parameters and the relationship of these changes to the physical systems. Therefore, even though the relative rotamer energies may not be very accurately known, variations in the rotamer energies can be carefully assessed.

Variations in the Vicinal Coupling Constants and Properties of the Mixed Aqueous Solvents. The changes in the vicinal coupling constants as they relate to variations in the rotamer energies can be conveniently discussed in terms of the quantity, $\Delta J = J_{13} - J_{12}$, the difference in the two coupling constants. ΔJ measures the divergence of the coupling constants and according to eq 1 can be expressed as a linear function of $(c - b)$, the difference in the populations of the C and B rotamers. Therefore, it takes into account variations in two of the populations and provides a more general description of the system than could be obtained by either of the coupling constants individually.

The variations in the rotamer energies observed here are apparently the result of variations in (the as yet imprecisely defined) solute-solvent interactions. Such interactions are likewise responsible for many of the bulk properties of these mixed solvent systems. In this section, three of these properties will be discussed in relation to variations in ΔJ . These properties are the solubility of phenylalanine in the mixed solvents, their dielectric constants, and the free energy of transfer of the phenyl group from water into the mixed solvents. The first and last properties are intimately connected with the effects of solute-solvent interactions; the dielectric constant is a general measure of the polar nature of the solvent, indicative of the kinds of solvent interactions possible.

A number of the appropriate data, especially the dielectric constants, are readily available in the literature. Data on solubilities are more limited and it was necessary to measure them directly for the acetone-, acetamide-, and dimethyl sulfoxide-water systems. These results and the pertinent literature information are collected in Table II.¹³⁻²⁰ Details of the measurements can be found in the Experimental Section.

The solubility of phenylalanine in the mixed solvents of composition 0.25 mol fraction in the nonaqueous component is plotted in Figure 4A vs. the phenylalanine anion ΔJ appropriate to the same solvents. The values of ΔJ are taken from Table I at 28° for all solvent systems except ethylene glycol-water for which the temperature is 52°. The value for pure water was interpolated from the data in ref 4a for 25°.

As illustrated by Figure 4A, the increasing divergence of the coupling constants roughly correlates with decreasing phenylalanine solubility. The point for water falls well below, while the point for acetone rises above the trend established by the other points. Nevertheless, despite the inexactness of the relationship, these results suggest that the solute-solvent interactions responsible for the increase in solubility of the phenylalanine molecule are related to the solute-solvent interactions that enhance the stability of the A and B rotamers.²¹

TABLE II: Amino Acid Solubilities and Dielectric Constants for the Mixed Aqueous Solvents

Solvent ^a	Solubility ^b		Dielectric constant ^c
	Alanine	Phenylalanine	
Water	0.0326 ^f	0.00302 ^f	78.5 ^j
Ethylene glycol	(-552) ^d	0.00320 ^g	61.9 ^k
Urea	(-740) ^e	0.00857 ^h	100.4 ^j
Methyl alcohol	0.0116 ⁱ	0.00232 ⁱ	60.9 ^k
Acetone	0.00423	0.00193	47.0 ^k
Acetamide	0.00709	0.00412	71.3 ^l
Dimethyl sulfoxide	0.00163	0.00092	73.6 ^m

^a Composition of the mixed solvents is 0.25 mol fraction in the non-aqueous component. ^b In mole fraction units at 25°. Entries without a reference are experimental results (see text). ^c At 25° unless stated otherwise. ^d Free energy of transfer of the phenyl group interpolated directly from the data in ref 13 (in cal/mol). ^e Free energy of transfer of the phenyl group extrapolated directly to 10.0 M from the data in ref 6 (in cal/mol). ^f Reference 13. ^g Reference 14. ^h Reference 15. ⁱ Reference 16. ^j Reference 17. ^k Reference 18. ^l Reference 19. ^m Reference 20.

Dielectric constant vs. ΔJ is illustrated in Figure 4B. A general but inexact correlation is found between decreasing ΔJ and increasing dielectric constant. It appears that the more polar the solvent mixture, the more are enhanced the A and B rotamer stabilities. This interpretation is consistent with the observed temperature and concentration dependence of the rotamer energies of the phenylalanine anion in pure water.^{4b} There, also, increases in the dielectric constant of the solution brought about an increase in the stability of the A and B rotamers. The lack of a more precise correlation here precludes a more quantitative development at this time.

Finally, ΔJ is compared to the free energies of transfer of the phenyl group in Figure 4C. The values are calculated from the following^{6,13}

$$\delta g_{tr}(\text{phenyl group}) \approx RT \left[\ln \frac{N_{PA,W}}{N_{PA,S}} - \ln \frac{N_{A,W}}{N_{A,S}} \right] \quad (3)$$

where $N_{i,j}$ is the concentration in mole fraction units of substance i in a saturated solution in solvent j and where PA, A, W, and S refer to phenylalanine, alanine, water, and mixed solvent, respectively. Activity coefficient corrections have been ignored in our calculations because the differences between estimates for phenylalanine and ala-

- (12) J_g and J_t are assigned values of 2.60 and 13.56 Hz, respectively. Cf. K. G. R. Pachler, *Spectrochim. Acta*, **20**, 581 (1964). Representative values obtained for the relative rotamer energies can be found in ref 4. The limits of validity of this interpretation are discussed later.
- (13) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **240**, 3568 (1965).
- (14) Linearly interpolated from the data in ref 13.
- (15) Value obtained by extrapolation to 10.0 M from the data in ref 6.
- (16) T. E. Needham, A. N. Paruta, and R. J. Gerraughy, *J. Pharm. Sci.*, **60**, 258 (1971). Input for the equations in M/l obtained from density data given in "Handbook of Chemistry and Physics," 47th ed, Chemical Rubber Publishing Company, Cleveland, Ohio, 1966.
- (17) J. Wyman, *J. Amer. Chem. Soc.*, **55**, 4116 (1933). Figure for urea-water system obtained by linear interpolation.
- (18) G. Akerloff, *J. Amer. Chem. Soc.*, **54**, 4125 (1932). Values obtained by linear interpolation.
- (19) J. Wyman, *Chem. Rev.*, **19**, 213 (1936). Result calculated assuming a linear decrement for a 9.1 M solution.
- (20) Y. Doucet, F. Calmes-Perrault, and M. T. Durand, *C. R. Acad. Sci.*, **260**, 1878 (1965); at 27°.
- (21) It should be pointed out that the precision of the relationship obtained depends on the units chosen for the solubility. Had the solubility been expressed in moles/liter of solvent or grams/100 grams of solvent, the correlation would have been somewhat improved. However, since there is as yet no well-founded theoretical justification for preferring one system of units over the other, the figure in its present form suffices to show the general trend.

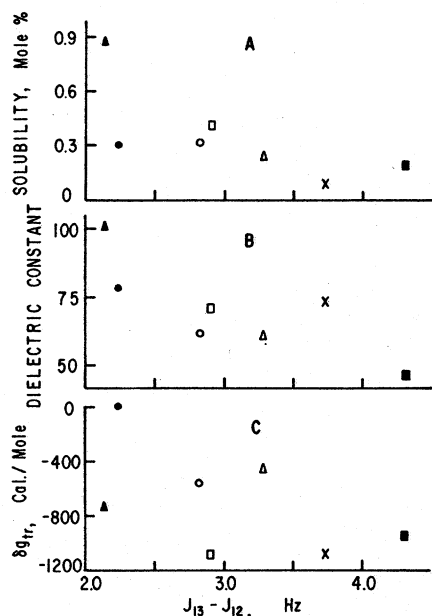


Figure 4. Comparisons of ΔJ with properties of the mixed aqueous solvents of composition 0.25 mol fraction: ▲, urea; ●, water; ○, ethylene glycol; □, acetamide; △, methanol; ×, dimethyl sulfoxide; ■, acetone. (A) ΔJ vs. the solubility of phenylalanine in the mixed solvents. (B) ΔJ vs. the dielectric constants of the mixed solvents. (C) ΔJ vs. the free energy of transfer of the phenyl group from water into the mixed solvents.

nine in urea⁶ and ethylene glycol¹³ solutions are sufficiently small (3–30 cal).

The results illustrated in Figure 4C show no regularity whatever. It was anticipated that the free energy of transfer of the phenyl group, which can be considered a measure of the "water-like" nature of the mixed solvent systems, would show a negative correlation with ΔJ . The expected interpretation would have been that the enhanced stability of the A and B rotamers (decreasing ΔJ) would be associated with water-like solute-solvent interactions connected with water structure. The results obtained

here, however, suggest that the interactions influencing the rotamers are not simply related to hydrophobic interactions.

Conclusion

The nonaqueous solvents appear to disrupt or diminish the solute-solvent interactions favoring the A and B rotamers of the phenylalanine anion in aqueous solution at low temperature, the intensity of the effect varying with the particular solvent employed. These results are closely related to those observed with studies of the concentration dependence of the nmr spectrum of the phenylalanine anion in pure water,^{4b} the addition of nonaqueous component having a similar effect as an increase in the concentration of the anion in pure water. Both decrease the ratio of the number of water molecules available *per* phenylalanine anion molecule. The degree of the enhancement of the A and B rotamer stabilities undoubtedly is intimately connected with the solvation of the rotamers which in turn depends on this ratio. The nonaqueous solvents present the additional capability of themselves participating in the solvation presumably as a function of whether they are more or less water-like. It had been anticipated that the nmr results would have displayed regularities with the properties of the aqueous solutions which could be used as a basis for developing these concepts. However, the lack of a precise relationship with both the dielectric and solubility properties and the lack of any clear relationship with the free energies of transfer indicate that the solvation must be represented in a highly complex fashion. It is probable that major advances in the theoretical understanding of solute-solvent interactions in aqueous media will be required in order to explain adequately these phenomena. Hopefully, the rotational isomerism of amino acids will provide a convenient framework against which to test such developments.

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